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Rapid Cycle DNA Amplification

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DNA amplification requires temperature cycling of the sample. From the viewpoint of the sample, the only relevant characteristics of a temperature cycler are its speed and homogeneity. How fast the sample temperature can be changed largely determines the cycle time. How uniform the sample temperature is affects reproducibility. As cycle speed increases, it becomes harder to maintain homogeneous temperatures within and between samples. Standard commercial instrumentation usually completes 30 cycles (94, 55, 74°C) in about 2–4 hr. A new "high-performance" system requires about half as much time and is reported to run two temperature profiles (60, 94°C) in a little over an hour (Haff et al., 1991). "Rapid cycle DNA amplification" as used here refers to completion of 30 cycles of amplification in 10–30 min. The physical (denaturation and annealing) and enzymatic (elongation) reactions of DNA amplification occur very quickly; with the proper instrumentation, amplification times can be reduced an order of magnitude from prevailing protocols.

Initial Development

Before commercial temperature cycling instrumentation was available, we started work on a thermal cycling system using capillary tubes with hot air temperature control (Wittwer et al., 1989). Our original instrument was designed to approximate the temperature profile and amplification of 100-μl samples in microfuge tubes transferred between water baths. Because of the low heat capacity of air, the thin walls and high surface area of capillary tubes, we realized that small volume samples could be cycled much faster than this if high velocity air were blown past the tubes. What effect rapid cycling would have on amplification was not known.

We modified our instrument for faster cycling of 10-μl samples (Wittwer et al., 1990). Total amplification times for 30 cycles were reduced to 10 min, and still specific amplified product was observed on ethidium bromide stained gels. Since there are six temperature/time variables in a typical three-temperature amplification protocol, what is the effect of changing each variable systematically? This

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was difficult to determine previously because of long transition times between temperatures in other instruments.

Cycle Optimization

The optimal times and temperatures for the amplification of a 536-bp fragment of β-globin from genomic DNA were determined (Wittwer and Garling, 1991). Amplification yield and product specificity were optimal when denaturation (93°C) and annealing (55°C) times were less than 1 sec. There was no advantage to longer denaturation of annealing times, as long as the DNA was heat denatured before temperature cycling. Yield increased with longer elongation times, although there was little change above 10–20 sec. These results may be surprising, but merely reflect the poor match between most commercial instrumentation for DNA amplification and the physical/enzymatic requirements of the reaction.

Figure 15.1 compares four different sample temperature/time profiles and their resultant amplification products after 30 cycles. Profiles A and B were obtained on a standard heating block/microfuge tube system. The transitions between temperatures are slow and many nonspecific bands are present. Some of the nonspecific bands can be eliminated by limiting the time at each temperature (A vs B) within the limits of the instrument. Profiles C and D were obtained with the rapid air cycler. Amplification is specific, and although yield is maximal in C (60 sec elongation), it is entirely adequate in D (10 sec elongation).

The importance of annealing time on product specificity is systematically studied in Figure 15.2A. As annealing time increases, so do spurious, undesired amplification products. Not only is the time spent at annealing important, but long transition times (25 vs 9 sec) also increase nonspecific amplification, probably because of more time spent near the annealing temperature.

We have now amplified over 50 primer pairs by rapid cycling and the method seems generally applicable. We have never observed any advantage to extending denaturation times beyond the minimum possible, as long as template DNA is denatured before cycling. The best specificity is obtained with minimal annealing times, although in some amplifications there appears to be a tradeoff between specificity and yield. Using minimal denaturation and annealing times is convenient because it reduces the total number of temperature cycling variables for optimization from six to four. If a constant denaturation temperature of 94°C is used (DMSO may be needed for high GC sequences), only three variables remain. In most cases, an elongation temperature of 70–74°C works well. The exceptions in our hands are certain VNTR areas (Boerwinkle et al., 1989) and DNA extracted from paraffin blocks, both of which seem to amplify better at 65–70°C. Two variables remain, the elongation time and the annealing temperature. The elongation time seems loosely determined by the product length. Fragments around 100 bp usually require no specific elongation time; extension is apparently adequate during the transition from annealing to denaturation, even with rapid cycling (Fig. 15.2B). This extension during transition explains the recent popularity of two-temperature amplification; slower conventional cycling would provide more than enough time for the extension of much larger fragments. Elongation times of 5–20 sec are usually appropriate for fragments up to 500 bp; while up to 30 sec may be needed for a 1-kb amplification. Algorithms for the prediction of the remaining variable (annealing temperature) have been published (Rychlik et al., 1990), although their application to rapid cycling has not been thoroughly tested.

Figs. 15.1

Amplification Additives

DNA amplification is robust and a variety of buffers and additives can be used without serious effect. These include Ficoll and electrophoresis indicator dyes to simplify gel loading directly out of capillary tubes (Wittwer and Garling, 1991). Bovine serum albumin seems to be most effective in preventing surface denaturation of the polymerase on glass capillary walls. Ethidium bromide can also be included

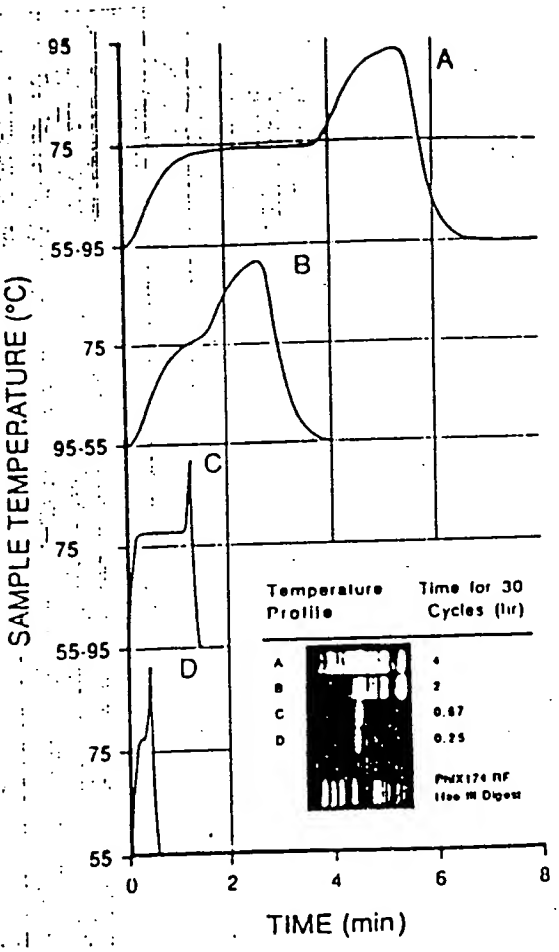

FIGURE 15.1. Comparison of standard heat block instrument (A and B) to rapid cycling instrumentation (C and D). Temperature–time profiles and amplification products obtained after 30 cycles of amplification of a 536-bp β-globin fragment from genomic DNA. Ten-microliter samples were electrophoresed through 1.5% agarose and stained with ethidium bromide. Sample temperature was monitored with a 0.2-mm-diameter thermocouple probe (IT-23, Sensortek, Clifton, NJ). Profiles A and B were obtained with 100-μl samples in microfuge tubes overlaid with 60 μl of mineral oil as recommended by the manufacturer (Perkin-Elmer Cetus DNA Thermal Cycler, Norwalk, CT). In A, a typical protocol is shown (heating block at 93°C for 1 min, 55°C for 2 min, and 74°C for 3 min). In B, the times were reduced so that the sample just momentarily reached denaturation and annealing temperatures (block at 55°C for 35 sec, 77°C for 45 sec, and 92°C for 35 sec. Profiles C (1 min elongation) and D (10 sec elongation) were obtained on 10-μl samples in capillary tubes in a custom rapid air cycler (Wittwer et al., 1990). Reprinted with permission from *BioTechniques* (Wittwer and Garling, 1991).

at concentrations used for staining gels (0.5 μg/ml) without apparent change in yield or specificity (Fig. 15.2C). This is surprising because of the known influence of ethidium bromide on DNA melting (Maeda et al., 1990). The potential for using ethidium bromide fluorescence during amplification as a monitor of double stranded DNA production is attractive. Figure 15.2D shows the relative fluorescence of reactions in capillary tubes before and after amplification. The capillary tubes were directly placed on a standard UV transilluminator. Although viral and plasmid amplifications can be monitored this way (with <5 ng starting DNA/10 μl reaction), background fluorescence from the larger amounts of DNA used in genomic amplifications (50–100 ng/10 μl) is quite high. With DNA nearly doubling each elongation step, direct measurement of the efficiency of each cycle might be possible. The end of elongation might even be triggered dynamically after a certain efficiency was achieved. Fluorescence monitoring could also control the number of cycles needed to achieve the fluorescence level for a particular yield. The effect of ethidium bromide on base incorporation error rate during DNA amplification has not been studied.

Capillary Tubes versus Microfuge Tubes

The microfuge tube is the standard small container in molecular biology. However, it is a poor container for rapid temperature cycling. This is only partly because of wall thickness: GeneAmp™ tubes have a wall thickness of 0.51 mm, MicroAmp™ tubes, 0.30 mm, and standard glass capillary tubes, 0.20 mm. Just as important, the geometry of samples in conical tubes limits the available surface area for heating. The rate of heat transfer at any surface is directly proportional to the surface area. The sample geometry in microfuge tubes is not easily described, but it approaches a cone with its tip truncated by a hemisphere. For any given volume, a sphere has the lowest surface area. In contrast, both cylinders and sheets can

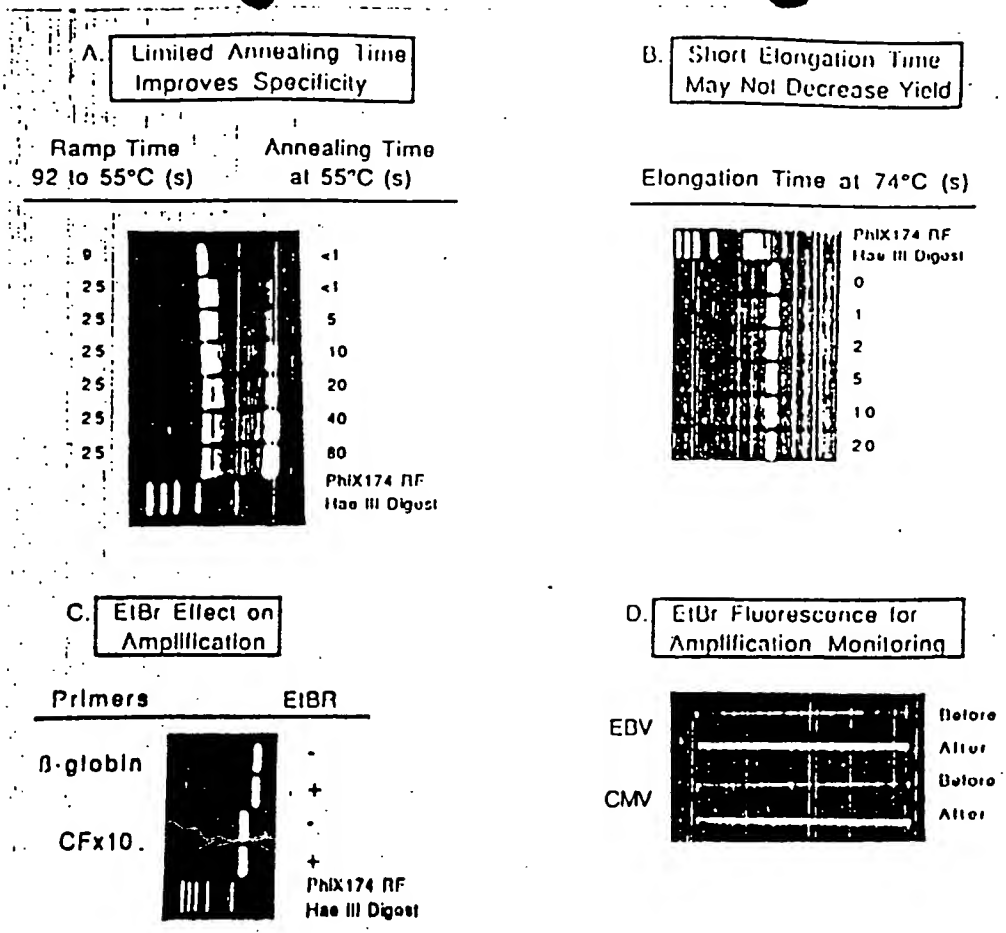

FIGURE 15.2. Amplification by rapid cycling: parameter optimization and use of ethidium bromide. (A) The effect of annealing time on the specificity of a β-globin fragment (536 bp) amplified from primers RS42 and KM29. Reprinted with permission from *BioTechniques* (Wittwer and Garling, 1991). (B) The effect of varying elongation time on the yield of product (149 bp) from primers Y1.1 and Y1.2 (Kogan et al., 1987). Thirty cycles of amplification from 50 ng genomic DNA were performed with settings of 0 sec at 94°C, 0 sec at 55°C, and the time indicated at 74°C. At 0 sec elongation, the total amplification time was 98 min. (C) The presence of 0.5 μg/ml ethidium bromide in the amplification reaction does not seem to alter specificity or yield. The β-globin fragment was amplified from 50 ng genomic DNA and primers PC03 and PC04 (Saiki et al., 1985) by 35 cycles of 0 sec at 94°C, 0 sec at 55°C, and 10 sec at 94°C. Exon 10 of the cystic fibrosis gene (Riordan et al., 1989) was amplified from 50 ng genomic DNA and primers GACTTCACTTCTAATGATGA and CTCTTCTAGTTGGCATGCTT) by 40 cycles of 0 sec at 94°C, 0 sec at 45°C, and 10 sec at 74°C. (D Photograph of UV transilluminated reaction mixtures containing 0.5 μg/ml ethidium bromide in capillary tubes before and after amplification. Five nanograms of DNA isolated from Epstein-Barr virus (EBV) or cytomegalovirus (CMV) cultures was cycled 30 times at 0 sec at 94°C, 0 sec at 50°C, and 10 sec at 74°C. A custom rapid hot air cycler (Wittwer et al., 1990) was used in A, with reactant concentrations as given previously (Wittwer and Garling, 1991). The Idaho Technology 1605 thermal cycler (Idaho Falls, ID) was used for all other amplifications.

be made to have an arbitrarily high surface area for any given volume if there is no restriction on the long dimension(s). A planar configuration would be appropriate for *in situ* amplification, for instance, between two microscope slide covers. A long cylinder (capillary tube) is more attractive for ease of sample addition and removal in routine DNA amplification.

The new "high-performance" amplification system (Haff et al., 1991) is probably the ultimate in heat-block, conical-tube amplification instruments. Its engineering is exquisite, but the technology is not well matched to rapid cycling as described here. A 10-μl sample in a MicroAmp™ tube has less than 25% of the surface area of a 10-μl capillary tube (Fig. 15.3A vs D). Although the conical portion of the tube is in direct contact with the heating block, the hemispherical tip appears insulated from it by a pocket of still air. With the small sample volumes optimal for rapid cycling, much of the sample surface area is insulated rather than heated. It is also more difficult to predict the temperature of small samples because of a lower "thermal time constant" (Haff et al., 1991). Samples are said to be rapidly mixed by convection. Convection is caused by temperature differences within the sample, a problem seldom discussed but sure to affect amplification, at least rapid cycle amplification. Temperature gradients become more difficult to control and more important as the cycle time decreases. The temperature variation throughout a 10-μl sample in a commercial rapid air cycler (1605 Air Thermo-cycler, Idaho Technology, Idaho Falls, ID) is ± 1°C at all time points of a 30-sec cycle, measured by moving a 0.2-mm-diameter thermocouple through the sample in a 0.5-mm-diameter i.d. capillary tube. We do not know the within-sample temperature variation in other systems. It is not easy to measure because of the small volumes involved. Nevertheless, temperature gradients significant enough for "rapid mixing by convection" apparently occur. Finally, the realization that denaturation and annealing times can be reduced to a minimum is not widely recognized. Sophisticated control algorithms have been developed to carefully approach and hold these temperatures without overshoot. Since denaturation and annealing temperatures do not need to be held, the transition to these temperatures can be at the maximal heating or cooling rate of the system. The next heating or cooling step can be triggered immediately when the target temperature is reached. This significantly speeds up cycle time by turning the denaturation and annealing "plateaus" into temperature "spikes" (Fig. 15.1).

Figure 15.3 compares the scale of different tubes and their obtainable temperature/time profiles with a 10-μl sample. A custom air cycler (Wittwer et al., 1990) was adjusted for temperatures of 93, 55, and 75°C with a 10 sec hold at elongation for each tube type. As the diameter of the tube increases, transition times and the total cycle time also increase. Although we cannot easily monitor conical tubes in our air cycler, some idea of the deterioration in temperature response with more globular samples is apparent.

Fig. 15.3

Even though capillary tubes have many advantages for rapid cycling, many people initially have difficulty using them. Accessories for holding, handling, and labeling capillary tubes are not as developed as for microfuge tubes. Samples are best mixed in microtiter plates with U-shaped wells and can be quantitatively loaded by capillary action, assisted by tilting the plates. Although yield may sometimes be increased by siliconizing the tubes (SigmaCote™ or others), this eliminates capillary action and is not routinely necessary. Capillary tubes holding up to 20 μl are available precut to length and are made of soft glass that can be readily sealed, not only with a torch or Bunsen burner, but with a cigarette lighter or candle. A candle is most convenient, although shielding from room air turbulence is necessary. Labeling, ordering, and handling the tubes have been problems. A simple device that holds eight capillary tubes at once at microtiter spacing is very useful. With this device, eight samples can be simultaneously loaded from a microtiter tray by capillary action, sealed, and cycled. After cycling, the ends of the tubes can be scored with a file and individually emptied into the wells of an analysis gel with a microaspirator. Sapphire or ce-

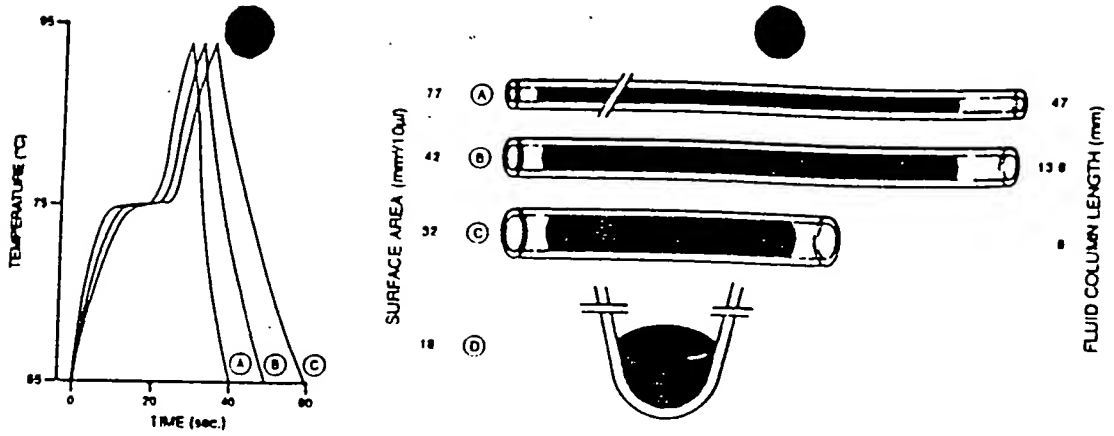

FIGURE 15.3. Effect of sample container on temperature response. Temperature–time tracings and scale drawings of 10°l samples in three different capillary tubes. The tubes were Kimble KIMAX (Vineland, NJ) #46485-1 (A, 10 µl volume at 4.7 cm), #46485-15 (B, 34 µl volume at 4.7 cm), and #34500-99 (C, 59 µl volume at 4.7 cm). A scale drawing of a 10-µl sample in a MicroAmp™ tube of the Perkin-Elmer Cetus GeneAmp PCR System 9600 (Haff et al., 1991) is shown for comparison (D).

ramic glass cutters last longer than diamond-coated triangular files that tend to lose their edge. All of these items mentioned, as well as prescored capillary tubes are now commercially available (Idaho Technology).

Rapid Cycle Instrumentation

Early custom-made hot air cyclers have been previously described (Wittwer et al., 1989, 1990). They can be envisioned as temperature-controlled recirculating hair dryers. For rapid cooling, the circular air path is broken and room temperature air introduced. Air is unique as a heat transfer medium; it is inexpensive, readily available, easily mixed, and never makes a mess. If it is rapidly blown past a high surface area-to-volume sample, heat transfer is rapid. For rapid cycling, air velocities of about 1000 m/min are used. This is in contrast to the convection oven design of some "stirred air" thermal cyclers that actually have slower cycle times than heat block instruments.

The commercial rapid cycling instrument (Fig. 15.4) has several unique features that distinguish it from earlier custom rapid cyclers. The sample temperature is no longer monitored by a delicate miniature thermocouple in a mock sample (that tends to evaporate). A permanent tubular thermocouple probe is precisely matched in temperature response to the sample in a capillary tube that can hold up to 20 µl. Although instrument settings can be modified to amplify higher volumes in larger diameter tubes, temperature transition rates increase and the advantage of rapid cycling decreases (Fig. 15.3). With larger tubes, denaturation and annealing times must be held for several seconds to allow the sample temperature to catch up to the air temperature. This is similar to the problem of matching the sample and block temperatures in heat block instruments; we do not recommend larger tubes. Instead of a nichrome wire coil as a heat source, a 500-W halogen light bulb is used. The bulb irradiates the surface of a cylindrical sample chamber that is lined with a light-absorbing, heat-stable foam, that in turn heats the circulating air and the sample tubes (Fig. 15.4). The air flow pattern in the commercial instrument has also been modified for more uniform air speed and temperature.

Fig. 15.4

Besides DNA amplification, rapid cycling should also be useful in cycle sequencing, and other amplification protocols such as the ligase

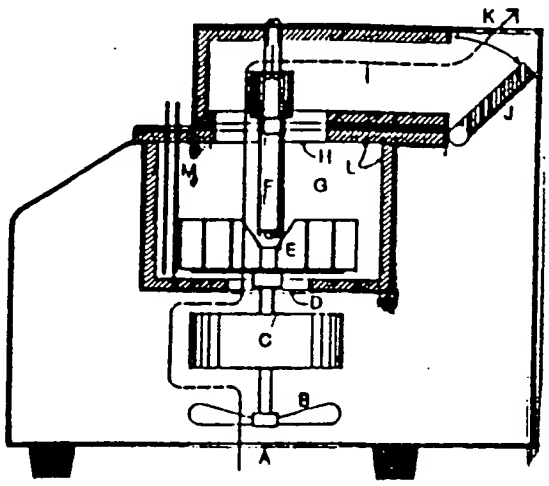

FIGURE 15.4. Schematic cross section of the Idaho Technology 1605 thermocycler. Samples in capillaries M are placed in cyclindrical air chamber G with turbulence created by fan blades E. Halogen lamp F irradiates the surface of chamber G, which is lined by light-absorbing foam L. Foam L heats the turbulent air, which in turn heats the samples in the capillaries. Room temperature air enters the instrument through vent A propelled by blade B. When the solenoid-operated door J is closed during heating, air exits through vent K without entering the sample chamber G or top chamber I. The top of chamber G contains top vent H of larger radius than bottom vent D, tending to draw air up through the sample chamber. However, no significant air can flow up through the chamber while door J is closed. The dotted line indicates the air flow path during cooling, initiated by opening door J. Cooling air flows around the fan motor C, through vent D, sample chamber G, vent H, top chamber I, and out through vent K. Rapid mixing of the air in chamber G by the fan blade E ensures spatial temperature homogeneity. All elements in the sample chamber are symmetric around the central vertical heating element (patents pending University of Utah/Idaho Technology).

chain reaction. Required times can be significantly reduced and specificity increased with rapid cycling. It is clear from Figure 15.2B that even current rapid cycle instrumentation may be too slow for the reactions comprising DNA amplification. Cycle times can be decreased further. More compact instruments can be built. Direct coupling to sensitive analysis systems is feasible. Rapid cycling only requires that the time-revered icons of heating blocks and conical tubes be left behind.

Recently, a report utilizing ethidium bromide for simultaneous amplification and detection in PCR appeared (Higuchi et al., 1992). Another report (Hoppe et al., 1992) described gel loading dyes compatible with PCR, although this had been previously reported (Wittwer and Garling, 1991).

References

Boerwinkle E, Xiong W, Fourest E, Chan L (1989): Rapid typing of tandemly repeated hypervariable loci by the polymerase chain reaction: Application to the apolipoprotein B 3' hypervariable region. *Proc Natl Acad Sci USA* 86:212-216.

Haff L, Atwood JG, DiCesare J, Katz E, Picozza E, Williams JF, Woudenberg T (1991): A high-performance system for automation of the polymerase chain reaction. *BioTechniques* 10:102-112.

Higuchi R, Dollinger G, Walsh PS, Griffith R (1992): Simultaneous amplification and detection of specific DNA sequences. *Bio/Technology* 10: 413-417.

Hoppe BL, Conti-Tronconi BM, Horten RM (1992): *BioTechniques* 12:679-680.

Kogan SC, Marie Doherty BS, Gitschier J (1987): An improved method for prenatal diagnosis of genetic diseases by analysis of amplified DNA sequences. *N Eng J Med* 317:985-990.

Maeda Y, Nunomura K, Ohtsubo E (1990): Differential scanning calorimetric study of the effect of intercalators and other kinds of DNA-binding drugs on the stepwise melting of plasmid DNA. *J Mol Biol* 215:321-329.

Riordan JR, Rommens JM, Kerem BS, Alon N, Rozmahel R, Grzelczak Z, Zielenski J, Lok S, Plavsic N, Chou JL, Drumm ML, Iannuzzi MC, Collins FS, Tsui LC (1989): Identification of the cystic fibrosis gene: Cloning and characterization of complementary DNA. *Science* 245:1066-1073.

Rychlik W, Spencer WJ, Rhoads RE (1990): Optimization of the annealing temperature for DNA amplification in vitro. *Nucl Acids Res* 18:6409-6412.

Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, Chang CA (1985): Enzymatic amplification of β-globin genomic sequences and

restriction site analysis for diagnosis of sickle cell anemia. *Science* 230:1350–1354.

Wittwer CT, Garling DJ (1991): Rapid cycle DNA amplification: Time and temperature optimization. *BioTechniques* 10:76–83.

Wittwer CT, Fillmore GC, Hillyard DR (1989): Automated polymerase chain reaction in capillary tubes with hot air. *Nucleic Acids Res* 17:4353–4357.

Wittwer CT, Fillmore GC, Garling DJ (1990): Minimizing the time required for DNA amplification by efficient heat transfer to small samples. *Anal Biochem* 186:328–331.